Relationship between Human Papillomavirus and Tumor Markers Expression among Women in Two Tertiary Hospitals in Bayelsa State, Nigeria

Oboma YI**, Ngokere AA¹ and Elesha S0³

¹Department of Medical Laboratory Science, Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria
²Department of Medical Laboratory Science, Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Awka, Nnewi Campus, Nigeria
³Department of Anatomical Pathology, Faculty of Basic Medical Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria

Abstract

Cervical Human Papillomavirus (HPV) infection in sub-Saharan Africa is among the highest in the world. HPV early proteins (E6 and E7) cause inhibition of p53 and Rb proteins respectively which are important for cell transformation. At S phase the proliferation can be achieved using various methods like polymerase chain reaction, immunohistochemistry/immunocytochemistry and Hybrid Capture 1/2. This study aimed at evaluating the effectiveness of some tumor markers in detecting cervical human Papillomavirus in HPV positive confirmed tissues by PCR. Fifty cervical tissue samples were subjected to nested polymerase chain reaction technique for the detection of HPV and immunohistochemistry method was used to localized and identify p53, p16 and Ki-67 antibodies. Result revealed 35.7% expression for p16, 58.8% for Ki-67 and 32.64% for p53 marker in the tissue blocks studied. Non-dysplastic cases expressed 62% for p16, 25% and 70% for Ki-67 and p53 respectively. Squamous Cell Carcinoma (SCC) and adenocarcinoma of the cervix showed 100 % expression for p16 gene, followed by p53 (91.8%) and Ki-67 (83.3%). Relationship between immunohistochemistry markers expression and cervical HPV using PCR was studied. Data obtained showed a 100% expression for p16, 53.8 % for Ki-67 and 30.8% for p53 tumor markers in all the HPV positive cases. HPV negative cases presented 59.5%, 67.6% and 81.7% expression for p16, Ki-67 and p53 respectively. All HPV positive cases showed statistically significant increase expression for p16 tumor marker compared with Ki-67 (P<0.001*OR=23.40) and p53 (P<0.001**, OR=41.73). These findings could be linked to retinoblastoma involvement in HPV infection which is the principle behind p16 staining reaction and therefore concluded that all HPV positive cases are p16 positive but not all p16 positive are HPV positive. We recommend p16 tumor marker as complementary test for HPV screening in poor resource centers.

Keywords: PCR; P16; HPV; IHC

Introduction

Cervical Human Papillomavirus (HPV) infection in sub-Saharan Africa is among the highest in the world just like cervical cancer [1]. A pooled analysis conducted by the International Agency for Research on Cancer (IARC) and HPV Prevalence surveys had reported the highest HPV prevalence in Nigeria [2,3]. Infection with the virus causes increase risk of cervical cancer, and once it’s get integrated into the human host genome it causes expression of viral mRNA protein referred to as early proteins E1 to E8. The E6 and E7 cause inhibition of p53 and Rb proteins respectively that are important for cell transformation into a dysplastic cell [4,5].

Tumor marker p16 is a cell cycle regulatory protein having tumor suppression function in cells with intact cell cycle through Retinoblastoma protein (Rb). It is a cell cycle dependent kinase inhibitor acting at S phase of the cell cycle [6]. Inactivated Rb protein causes elevated levels of p16 in hr-HPV infected cells and is expressed as a negative feedback control of functional Rb protein [7,8]. It can be detected by immunohistochemistry and immunocytochemistry; it gives a nuclear and cytoplasm staining. It is usually nuclear based in CIN-I and cytoplasm based in CIN-II/ III, invasive cancer and glandular intraepithelial neoplasia. The immunohistochemical reaction is occasionally positive in tubal metaplastic cells, squamous metaplastic cells, endocervical cells, giant cells, malignant/benign endometrial cells, atypical glandular cells, koilocytes and atrophic cells with minimal nuclear aberration [8]. It’s over expression is associated with E7 oncoprotein which inactivates retinoblastoma protein. This marker expression by IHC is superior to assay showing hr-HPV viral load only because p16 assess gene expression / alteration and not merely the presence of virus [8]. It is efficient triage to colposcopy in women with ASCUS/LSIL and finding / predicting the underlying HSIL. It has similar or slightly low sensitivity and specificity compared to HPV test, P16 is over (frequently) 100% expressed in HSIL, Squamous cell carcinoma, adenocarcinoma and 70-100% in LSIL [8]. It is utilized in the detection of hr-HPV than HC2, avoids unjustified anxiety and gives better correlation with subsequent tissue diagnosis as increase risk of subsequent HSIL in LSIL [6,8]. The p16 marker is a valued marker in follow-up group predicting HSIL more in association with Hybrid capture 2 positive for hr-HPV.

Ki-67 is a proliferative marker expressed in cells at all phases of the cell cycle except in G0 phase with maximum intensity during mitosis. It is an antigen marker for G1, S, G2/M phases [9]. It is a gold standard for proliferative index [8]. The marker can be demonstrated by IHC and it takes nuclear stain. In normal cervical epithelium, the expression is

*Corresponding author: Oboma, YI, Department of Medical Laboratory Science, Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University Wilberforce Island Bayelsa State, Nigeria, Tel: +2347030244521; E-mail: yoboma78@gmail.com

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seen only in basal and parabasal epithelium while increased expression is often seen in cervical dysplasia and carcinoma [10]. In cervical intraepithelial neoplasia cases, the expression is seen throughout the different epithelial layers and increases with different grade of the dysplasia independent of histology; It has a potential prognostic value and also valuable for follow up [8]. It is an additional prognostic marker and correlates with the histological grade of cervical carcinoma [11]. Higher expression is seen in tumors with a higher grade/stage at diagnosis associated with poor outcome and tumors with hr-HPV 16/18 infection compared with Lr-HPV [9,10].

Tumor suppressor gene (p53) plays a role in the control of cell cycle regulation through p21. The gene is located on chromosome 17 and monitors the integrity of the cell. Loss of p53 function is believed to play an important role in the pathogenesis of cervical cancer [12]. It may be inactivated as somatic point mutation, loss of heterozygocity, and by HPV oncoprotein E6, which binds wild type p53 and causes degradation by cellular ubiquitin pathway [13]. The p53arg is more efficiently inactivated by hr-HPV E6 protein than p53pro variant and women with p53arg are at higher risk of HPV associated cervical cancer than p53pro. Degradation and inactivation of p53 by hr-HPV E6/E6AP (associated protein) complex binding to central region of p53 is important in cervical carcinogenesis [13]. Other cellular proteins are implicated in the inactivation of p53 causing increased proliferation and decreased apoptosis [9,13]. The expression progressively increases with LSIL to HSIL. Squamous cell carcinoma and a few cases of adenocarcinoma compared with normal cervix [9]. Some authors on the contrary have found relative increased expression of p53 in CIN I compared to CIN II/ III which may be due to p53 degradation by HPV. However, according to Tan et al. [14] p53 does not give prognostic information and does not correlate with tumor recurrence.

Furthermore, HPV testing can be done using various methods like PCR (Polymerase Chain Reaction), immunohistochemistry/ immunocytochemistry, Hybrid Capture (HC) 1 and 2. Therefore, this study was aimed at evaluating the effectiveness of some tumor markers in the detection of cervical human Papillomavirus in formalin fixed paraffin embedded HPV positive confirmed tissues by PCR method.

**Methodology**

**Study area**

This study was a dual center study carried out in Niger Delta University Teaching Hospital - Okolobiri (NDUTH) and Federal Medical Centre Yenagoa both in Bayelsa State, South-South geopolitical zone of Nigeria. Both hospitals serve as referral centers in the state own University. The major occupation of the inhabitants is farming, fishing, palm oil milling, lumbering, palm wine tapping and local gin making.

**Immunohistochemistry Staining**

The methods of Nielsen et al. [15] and Oboma et al. [16] was adopted. Avidin Biotin Complex (ABC) method was applied on the Formalin Fixed Paraffin Embedded (FFPE) sections. The principle was based on the localization of antigens on the tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody reaction that is visualized by a marker enzyme, 3, 3 diaminobenzidine (DAB). The primary antibodies were antibodies specific for p53, p16 and Ki-67 antigens on the tissues and antibody dilution factor of 1:100 was used.

The formalin fixed paraffin embedded tissue were sectioned to 2 microns using Leica rotary microtome and then dewaxed at 70°C for 1hr. Sections were brought down to water by passing two changes of xylene, then 3 changes of descending grades of alcohol (Absolute 1%, 95%, and 70%) and finally to water. Antigen retrieval was performed on the sections by heating the slides on a citric acid solution PH 6.0 using the Microwave plate at 100°C for 15 min. The slides were equilibrated gradually with cool water to displace the hot citric acid for 5 min, and allowed to cool. Peroxidase blocking was done with 3% Hydrogen Peroxide (H2O2) for 15 min washed with Phosphate Buffered Saline (PBS) for 2 min. Protein blocking was performed using Avidin for 15 min. Sections were washed with PBS and endogenous biotin blocked using biotin for 15 min. After washing with PBS, sections were incubated with 1:100 dilution of the respective primary antibody (p16, p53 or Ki-67) for 60 min. Excess antibody washed off with PBS and secondary antibody applied on the section for another 15 min. Sections were washed and then labeled with the Horse Radish Peroxidase (HRP) for 15 min. A working DAB solution was prepared by mixing 1 drop (20 microns) to 1 mL of the substrate. This working solution was applied on sections after washing off the HRP with PBS for 5 min. The brown reaction begins to appear at this moment especially for a positive target. Excess DAB solution precipitates was washed off with distilled water. Sections were counterstained with Hematoxylin solution for 2 min and blued briefly. Sections were dehydrated in alcohol, cleared in xylene and mounted with DPX. Appropriate negative controls were prepared by eliminating the primary antibody step for p16 and Ki-67. Cells with specific brown colors in the cytoplasm, cell membrane or nuclei depending on the antigenic sites were considered to be positive. The stained cells without any form of brown colors were scored as negative. Nonspecific binding/artifacts on cells and connective tissue were disregarded. Quantification was as follows: Less than 5% of cells positive – Negative, 5 -24 cells positive - +1, 25 -49% cells positive -+2, 50 -70% cells positive +3 and 75-100% cell positive +4. The sections stained for ki-67 proliferation index were assessed as nuclear stain and evaluated using scores 0-3: Less than 10% cell positive – negative 10-30% cells positive= +1, 30-50% cells positive = +2 and more than 50% cells positive= +3(Figure 1).

**Detection of human papillomavirus using nested PCR**

The method of Entiauspe et al. [17] and modification of Oboma and Ngokere [18] was adopted for detection of human papillomavirus. MY09/11 and GP5+/6+consensus primers were used to amplify the extracted DNA in a two-step reaction. The MY09/11 consensus primer was used for the primary amplification while the GP5+/6+ consensus primer was used for the secondary amplification step. The PCR mixture for the primary amplification reaction included; 1x master mix (which contains Taq polymerase, dNTPs, MgCl2), forward and reverse primers at concentration of 0.2 μM and 5 μL of the extracted DNA used as template. Nuclease free water of 6.9 μL was used to make up the PCR components to a final volume of 25 μL for the primary amplification. The PCR conditions used was initial denaturing step at 94°C for 5 min, followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s and initial extension at 72°C for 60 s, with a final extension at 72°C for 10 min. The PCR mixture for the secondary amplification was 1x master mix, forward and reverses primers at concentration of 0.2 μM and 1 μL of the amplified product of the primary amplification as template. Nuclease free water of 11.5 μL was used to make up the PCR components to a final volume of 25 μL. The PCR condition used included; initial denaturing step at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 42°C for 60 s and initial extension at 72°C for 30 s, with a final extension at 72°C for 4 min.
The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Protein band sizes were estimated by comparison with 100 bp molecular weight marker (Quick-Load DNA molecular ladder, New England Biolabs Inc.). Ethical clearance (NDUTH/REC/0003/2015) was obtained from the ethical committee of the above mentioned hospital (Figure 2).

**Result**

Table 1 presented consensus primers used for the human Papillomavirus DNA amplification. MY09/MY11 with a band size of 450 bp was use for the primary reaction and GP5+/6 of 120 bp for secondary reaction step. Data from the 50 formalin fixed paraffin embedded cervical tissue samples analysed showed total tumour marker expression of 70%, 68% and 36% for p16, Ki-67 and p53 respectively as shown in Table 2. The percentage expression in non-dysplastic cases for p16, Ki-67 and p53 was 62.0%, 25.0% and 75.0% respectively with the highest seen in p53. Squamous Cell Carcinoma (SCC) and adenocarcinoma (ADC) of the cervix (dysplasia) showed 100% over expression for p16 gene, followed by 91.6% for P53 and 83.3% for Ki-67.

**Figure 1:** Gel agarose electrophoresis showing HPV detection using MY09/MY11 and GPS+/6+ primers.

**Figure 2:** Tumor markers labeling using immunohistochemistry stain.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Forward and revere primers sequence</th>
<th>Size Bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY09/11</td>
<td>CGTCMARRGGGAWACTGATC</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>GCMCAGGGWCATAYAAGG</td>
<td></td>
</tr>
<tr>
<td>GP5+/6+</td>
<td>TTGTGACTGTTTAGATACTAC</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>GAAAAATAACGTATATAATATCC</td>
<td></td>
</tr>
</tbody>
</table>


**Table 1:** Primers synthesis.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>% (N)</th>
<th>+P16 expression</th>
<th>+Ki-67 expression</th>
<th>+P53 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non dysplasia</td>
<td>08(100)</td>
<td>02(62.0)</td>
<td>05(25.0)</td>
<td>06(75.0)</td>
</tr>
<tr>
<td>Endometrial hyperplasia</td>
<td>10(100)</td>
<td>06(60.0)</td>
<td>08(80.0)</td>
<td>05(50.0)</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>10(100)</td>
<td>07(70.0)</td>
<td>05(50.0)</td>
<td>06(60.0)</td>
</tr>
<tr>
<td>SCC</td>
<td>12(100)</td>
<td>12(100)</td>
<td>10(83.3)</td>
<td>5(41.0)</td>
</tr>
<tr>
<td>ADC</td>
<td>03(100)</td>
<td>03(100)</td>
<td>02(66.6)</td>
<td>01(33.3)</td>
</tr>
<tr>
<td>Others</td>
<td>07(100)</td>
<td>05(71.4)</td>
<td>05(71.4)</td>
<td>05(71.4)</td>
</tr>
<tr>
<td>Total</td>
<td>50(100)</td>
<td>35(70.0)</td>
<td>32(64.0)</td>
<td>28(56.0)</td>
</tr>
</tbody>
</table>

SCC: Squamous Cell Carcinoma; ADC: Adenocarcinoma

**Table 2:** P16, ki-67 and p53 genes expression among cervical lesions studied.
Relationship between immunohistochemistry (IHC) tumour expression and cervical HPV using PCR was presented in Table 3. Of the 37 FFPE samples that were HPV negative by PCR, 22 of the samples expressed p16 gene. Also, out the 13 FFPE blocks that were HPV positive by PCR, p16 showed 100% expression, Ki-67 (53.8%) and p53 (30.8%).

In the present study, observed that patients with Squamous cell carcinoma have decreased p53 expression, compared with those with cervicitis. And this was in contrast to previous studies that documented no significant difference in p53 expression between CIN I, CIN II and CIN III [31]. Though some studies have established p53 increases proportionally to the grade of CIN and cervical cancer [32]. Similarly, Bibbo et al. [33] reported that there seems to be a significant correlation between p16 and Ki-67 immunoreactions among participant with advanced cervical lesions. Queiroz et al. [34] have equally assessed the expression of p16, Cyclin D1, p53 and Ki-67 proteins, and found greater lesion progression correlation for p16 and Ki-67 than for p53 and Cyclin D1.

### Table 3: Relationship between tumor expression and HPV positive samples.

<table>
<thead>
<tr>
<th>HPV status</th>
<th>p16 expression</th>
<th>Ki-67 expression</th>
<th>p53 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV positive</td>
<td>13(100)</td>
<td>13(100)</td>
<td>07(53.8)</td>
</tr>
<tr>
<td>HPV negative</td>
<td>37(100)</td>
<td>22(59.5)</td>
<td>29(76.8)</td>
</tr>
<tr>
<td>Total</td>
<td>50(100)</td>
<td>35(70.0)</td>
<td>32(64.0)</td>
</tr>
</tbody>
</table>

### Table 4: Comparison of P16 tumor marker Performance index in predicting HPV in tissue section compared with Ki67 and p53 markers.

<table>
<thead>
<tr>
<th>Tumor expression</th>
<th>Positive expression</th>
<th>Negative expression</th>
<th>Fisher exact</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16</td>
<td>13</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>7</td>
<td>6</td>
<td>P&lt;0.00144, OR=23.40, 95%ci= 1.15-475.9</td>
</tr>
<tr>
<td>P53</td>
<td>5</td>
<td>8</td>
<td>P&lt;0.0016*, OR=41.73, 95%ci=2.03-855.3</td>
</tr>
</tbody>
</table>

Figure 1 shows the HPV consensus primers used for the Viral DNA amplification while Figure 2 represent gel agarose electrophoresis showing HPV detection using MY09/MY11 and GP5+/6+ primers. Lane C represents the negative control and lane M represents the ladder Quick-Load 500 bp DNA molecular ladder. Furthermore Figure 2 presented photomicrographs of IHC positive slides in HPV positive samples. The brownish dot shows the presence of IHC positive sample.

### Discussion

**Immunohistochemistry markers and cervical lesions**

The p16 gene is a component of p16INK4a-Cdk4-6/Cyclin D-pRb signaling pathway. The present study revealed an increased expression of p16 with predominance in both nuclear and cytoplasm staining in the fifty (50) formalin fixed paraffin embedded cervical tissues subjected to immunohistochemistry staining (Table 2). However, the observed increased expression of p16 in the formalin fixed paraffin embedded tissues evaluated was lower compared to El-Hamdani et al. [19] who reported a higher percentage over expression in the cervical biopsies studied. Also, Milde-Langosch et al. [20,21] analyzed the presence of p16INK4a in cervical neoplasia and reported a relationship between p16INK4a expression and cervical neoplasia; raising hope that p16INK4a could represent a specific and sensitive marker for cervical neoplasia. The p16INK4a is also a negative regulatory protein that regulates the progression of eukaryotic cells through the G1 phase of the cell cycle and plays a central function in the regulation of cell cycle activation and is often perturbed in many cancers [22].

Tumor marker Ki-67 has been studied as an important proliferative tumor marker [23,24]. Its expression is related to proliferative action, lesion grade and human Papillomavirus infection [25,26]. The present study observed that patients with Squamous cell carcinoma had increased Ki-67 expression in multilayer Squamous epithelium compared to those with cervicitis and cervical hyperplasia as seen in Table 2. The finding is in agreement with previous work of Alessandra et al., [27] who reported that Ki-67 expressions in the basal cell layer present more often, with only a case presented with expression in intermediate layer. Further line of evidence buttresses the fact that that Ki-67 expression is restricted to the basal layer in normal epithelia [28,29]. In another study by Keating et al., [30], the highest proliferative activity measured by Ki-67, was found in high grade squamous lesions and is in line with the present study.

Although the immunohistochemical results for p53 protein detection in the cervix is still contradictory, the present study demonstrated that patients with Squamous cell carcinoma have decreased p53 expression, compared with those with cervicitis. And this was in contrast to previous studies that documented no significant difference in p53 expression between CIN I, CIN II and CIN III [31]. Though some studies have established p53 increases proportionally to the grade of CIN and cervical cancer [32]. Similarly, Bibbo et al. [33] reported that there seems to be a significant correlation between p16 and Ki-67 immunoreactions among participant with advanced cervical lesions. Queiroz et al. [34] have equally assessed the expression of p16, Cyclin D1, p53 and Ki-67 proteins, and found greater lesion progression correlation for p16 and Ki-67 than for p53 and Cyclin D1.

### Tumor marker expression and cervical human Papillomavirus

Higher percentage prevalence of p16 tumor marker expression was observed in Squamous cell carcinomas and adenocarcinoma samples positive for HPV by PCR in the present study. None of the HPV-positive samples studied were negative for p16 immunohistochemistry expression, therefore suggesting a higher specificity for HPV infection. These findings could be linked to retinoblastoma involvement in HPV infection which is the principle of p16 reaction. Klaes et al. [24] reported that p16INK4a is highly expressed in HPV induced Squamous carcinomas and adenocarcinoma agreeing with the finding of the current work. It was therefore concluded that HPV positive tumors are characterized by high expression of p16 and therefore should be considered as a surrogate marker for HPV in developing countries.

The present study observed that p16 and Ki-67 cocktail might be useful as an adjunct tool to predict the development of CIN lesions in women with normal or equivocal cytology requiring follow-up in Bayelsa State due to their affinity for hpv DNA. Yet it is important to note that p16 inactivation would not confer any further growth promoting effect. This is because in cervical cancer, the hr-HPV oncogenes E7 induces a permanent release of E2F from its binding to pRb, leading to continuous cell cycle activation [35] and thus the potential risk of developing a cervical lesion does not necessarily mean that HPV infection would progress [36,37].

Moreover, since transcription of the E7 oncogenes is required for p16INK4A up regulation, it has been suggested that carcinomas with increased expression of p16INK4A represents HPV induced tumors [24,38]. Although other pathways cannot be ruled out, increased expression of p16INK4A in the setting of CIN probably occurs mainly as a result of inactivation of retinoblastoma (Rb) by high-risk HPVs. The relationship between tumor markers (p16, p53 and Ki-67) and Human Papillomavirus (HPV) was established in the present study. There exist a statistically significant association between HPV infection and p16 expression compared with Ki-67 and p53 expression respectively. In the present study, most of the p16 positive samples were atypical lesions associated with high-risk HPV types with significant history of dysplasia, or had concurrent dysplasia. Interestingly, Sahebali et al. [23] and Sano et al. [39] have found a close correlation between the numbers of p16-stained cells and the HPV type and had concluded that intense staining was founded in HPV16 type and lowest in low-risk HPV lesions agreeing with the current findings.
Conclusion

This study was a dual center study and not population based with a limited number of cases analyzed and thus it is difficult to draw significant conclusions from this sample size. Yet, tumor marker p16 immunohistochemistry staining seems to be diagnostic and specific for human Papillomavirus induced cervical neoplasia. Therefore the use of p16 marker is suggested to compensate the lack of specificity of HPV testing using Pap and molecular methods. It is equally advocated to serve an adjunct tool in routine diagnosis of HPV in resource poor countries.

References